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Standardization in Bilirubin Assays: Evaluation of Selected Methods and Stability of Bilirubin Solutions

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Because of the inaccuracy of bilirubin (BIL) determinations, we have investigated some sources of error associated with the most commonly used methods. Inadequate standardization appears to be the most common error. BIL standards in either human serum albumin (HSA), bovine serum albumin (BSA), or pooled human serum were analyzed by these methods. Molar absorptivities (ϵ) of azobilirubin by the Jendrassik-Grôf procedure were practically identical in all three protein bases. The Meites-Hogg and Malloy-Evelyn methods gave substantially higher ϵ values with BIL in serum than with BIL in either HSA or BSA. The precision of all methods was good, but best with the Jendrassik-Grôf procedure. BIL standards can be prepared with good reproducibility (CV <0.5%). Standards deteriorate appreciably at −23 °C but are stable at −70 °C. Our data indicate a need for improved commercial BIL controls.

Additional Keyphrases: *sources of error in bilirubin determination • inter-method comparison • stability of bilirubin • bilirubin standards*

In 1960 Mather (*1*) made the statement that "Bilirubin determinations are perhaps the most notoriously unreliable of any in clinical chemistry," and they are still deplorable, although there has been considerable improvement since then. Serious problems associated with the determination of BIL[1] have been amply documented by the Comprehensive Clinical Chemistry Surveys of the CAP. Data from about 1 500 participating laboratories, compiled for the last few years, are shown in Table 1. What is considered as "acceptable performance" is definitely unacceptable, and what is called "good performance" cannot realistically fulfill the requirements and goals of a laboratory. "Acceptable performance" includes results within two standard deviations from the mean value, and "good performance" includes values within one standard deviation (1.5 standard deviation before 1970). The coefficient of variation is quite high and has not substantially decreased during the last four years. Further evidence of poor accuracy of BIL analyses is provided by the Proficiency Testing Program of CDC, Atlanta, Ga. BIL data reported in the Clinical Chemistry Summary Analysis for three months of 1972 are seen in Table 2. The lower and upper limits include the central 95% of all volunteer laboratory results. Without exception, the reported data failed to meet the "clinical requirements" limits set by CDC. These limits include results within one-fourth of the normal range (taken as 0.0-2.0 mg/dl) from the median value of the reference laboratories. The situation is worse when one considers results for a single sample analyzed by different methods (Table 3).

Table 1. College of American Pathologists' Comprehensive Chemistry Survey: Bilirubin, mg/dl

Year	Set	Mean	CV	"Good performance"	"Acceptable performance"
1968	3	13.5	13.8	10.7–16.3	9.8–17.4
	4	4.2	14.3	3.3–5.1	3.0–5.4
1969	C–1	15.2	10.1	12.9–17.5	12.1–18.3
	C–2	11.9	10.4	10.0–13.7	9.4–14.3
	C–3	19.6	10.5	16.5–22.7	15.5–23.7
	C–4	13.3	10.3	10.8–15.9	9.9–16.7
1970	C–A	2.6	15.3	2.2–3.0	1.8–3.4
	C–B	14.5	13.8	12.5–16.5	10.5–18.5
	C–C	15.8	14.6	13.5–18.1	11.2–20.4
1971	C–A	3.2	12.5	2.7–3.5	2.3–3.9
	C–C	3.9	10.3	3.5–4.3	3.1–4.7
	C–D	3.8	10.5	3.4–4.2	3.0–4.6

Lack of accuracy in any type of chemical analysis can be attributed to several factors, the most prominent being poor methodology, errors in standardization, and inadequate quality control. The BIL methodology has been extensively reviewed (*2, 3*). We do not propose here a new and better method, but our purpose is to evaluate the commonly used methods and possible sources of error associated with them.

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[1] Nonstandard abbreviations used: BIL, bilirubin; CAP, College of American Pathologists; CDC, Center for Disease Control; J-G, Jendrassik and Grôf; M-E, Malloy and Evelyn; ϵ, molar absorptivity; DMSO, dimethylsulfoxide; HSA, human serum albumin; BSA, bovine serum albumin; NBS, National Bureau of Standards; M-H, Meites and Hogg; and EDTA, ethylenediaminetetraacetate.

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Table 2. Center for Disease Control Proficiency Testing: Clinical Chemistry Summary Analysis (February, May, and November 1972)

	Bilirubin, mg/dl (all methods)							
	Volunteer laboratories			Reference laboratories			Clinical requirements	
Specimen	Lower limit	Median	Upper limit	Lower limit	Median	Upper limit	Lower limit	Upper limit
C 2007	2.8	4.1	5.6	2.4	3.9	6.0	3.4	4.4
C 2008	2.9	4.2	5.6	2.4	3.9	6.0	3.4	4.4
C 2009	2.4	3.4	4.3	2.2	3.2	4.8	2.7	3.7
C 2025	3.0	4.0	5.0	2.1	3.8	5.5	3.3	4.3
C 2026	1.8	3.8	5.1	1.3	3.8	5.4	3.3	4.3
C 2027	1.7	3.4	4.7	1.3	3.6	5.5	3.1	4.1
C 2028	1.7	3.5	4.8	0.8	3.6	5.0	3.1	4.1
C 2068	6.4	8.4	10.5	6.6	8.5	10.8	8.0	9.0
C 2069	3.0	4.4	5.6	3.3	4.4	5.7	3.9	4.9
C 2070	1.3	2.0	2.7	1.3	2.0	2.7	1.5	2.5

Table 3. Center for Disease Control Proficiency Testing: Clinical Chemistry Summary Analysis (Specimen C 2026, May 1972)

	Bilirubin, mg/dl					
Method (manual)	Volunteer laboratories			Reference Laboratories		
	Lower limit	Median	Upper limit	Lower limit	Median	Upper limit
Jendrassik–Gróf	3.2	4.3	5.2	3.8	4.1	4.3
Malloy–Evelyn	0.9	2.4	5.2	1.3	2.2	3.9
Spectrophotometric	2.2	3.1	6.0	–	–	–
Other	0.5	4.1	5.0	2.5	2.7	4.1

Data available from CAP Surveys and CDC Proficiency Testing indicate that 85 to 97% of the participants are using some modification of the J-G (*4*) or M-E (*5*) methods. Consequently, these two procedures and an attractive modification of the M-E method described by Meites and Hogg (*6*) were selected for evaluation.

Clinical chemistry laboratories have been plagued for years by unreliable BIL preparations and commercial reference sera. In 1962 a Joint Committee (*7*) accepted the ϵ of BIL in chloroform as a criterion of purity for an "acceptable BIL standard." The same Committee provided directions for the preparation of a BIL standard in serum. It is noteworthy that With (*8*) and Michaëlsson (*2*) have questioned the ϵ value of BIL in $CHCl_3$ as a criterion of purity, and suggested that the ϵ value of azobilirubin may be a more reliable criterion.

The problem of standardization in the analysis of BIL is quite complex and has several ramifications. Availability of pure and certified (by CAP) BIL has not improved the accuracy of the analysis, because of the variety of ways by which BIL standards have been prepared and used. The steps involved in the preparation of a BIL standard include: (*a*) solution of the material in a small volume of Na_2CO_3 (*7*), NaOH (*2*), cyanide-formamide (*9*), or DMSO (*10*); (*b*) addition of a protein base; (*c*) adjustment of pH to 7.3–7.4; and (*d*) dilution to a final volume, and storage.

Some BIL specimens dissolve with great difficulty in Na_2CO_3 solution. If the protein base is added before the solution is complete, the BIL will not dissolve, resulting in an erroneous standard. BIL dissolves easier in NaOH, but also degrades faster in this solvent. Solutions of BIL in DMSO must be added dropwise to the protein diluent with continuous stirring to avoid protein precipitation. Furthermore, quantitative transfer of BIL solutions in DMSO to the protein base is difficult. The importance of the protein base has been generally overlooked. Most authors have recommended fresh pooled serum (*2, 7, 9, 11*), others have used HSA (*12, 13*) or BSA (*10, 14*) solutions, and some investigators (*5, 15, 16*) preferred to carry out the standardization in a mixture of chloroform and methanol in the absence of protein. Because BIL is determined in serum or plasma, it is of paramount importance that for any chosen method the molar absorptivity of azobilirubin in HSA, BSA, or chloroform–methanol is equal to that in serum. The assumption that azobilirubin has the same ϵ value in the commonly used protein bases may not be valid.

The stability of frozen BIL preparations has not been conclusively established. Stored at −20 °C, BIL solutions are reported to be stable for at least 10 months (*14*), indefinitely (*17*), or for about a month (*9, 10, 18*).

Commercial control sera have been evaluated in the past (*15*), and recent reports by Helman et al. (*19*) and Laessig et al. (*20*) revealed that the quality of these controls is far from satisfactory. Many laboratories use commercial controls as reference BIL materials, especially with certain types of instrumentation such as multichannel continuous-flow systems. The validity of BIL analyses depends on the accuracy of these controls.

We have also obtained information regarding: (*a*) the precision of the preparation of BIL standards; (*b*) the ϵ values of azobilirubin in various BIL preparations; (*c*) the stability of BIL solutions at −23 °C and −70 °C; (*d*) the effect of the protein base and its concentration on the ϵ values of azobilirubin by the chosen methods; and (*e*) the precision of BIL analyses under optimum conditions.

Materials and Methods

Equipment

A Cary 16 spectrophotometer (Cary Instruments, Monrovia, Calif. 91016) and "Suprasil (QS)" 10 ± 0.01 mm cuvets (Helma Cells Inc., Jamaica, N. Y. 11424) were used for absorbance measurements. The photometric accuracy of the instrument was established and regularly checked by the use of standard glass filters (Standard Reference Material No. 930) available from the National Bureau of Standards. Transmittance values for these filters are certified with an uncertainty of ±0.5% of the nominal values, or 0.0022 absorbance units. The linearity of the instrument was checked with uranium-yellow filters obtained from Cary Instruments.

Materials

BIL samples purchased from J. T. Baker Chemical Co., Phillipsburg, N. J. 08865; Pfanstiehl Laboratories Inc., Waukegan, Ill. 60085; Harleco, Division of American Hospital Supply Corp., Philadelphia, Pa. 19143; and NBS (Standard Reference Material No. 916), Washington, D. C. 20234 were used without further purification. The following commercial BIL controls were examined: "Calibrate 3" and "Pediatric Versatol" (General Diagnostics Division, Warner-Lambert Co., Morris Plains, N. J. 07950), Dade "Bilirubin Control" and "Monitrol II" (Dade Division, American Hospital Supply Corp., Miami, Fla. 33152), "Metrix" (Armour Pharmaceutical Co., Clinical Diagnostics Division, Chicago, Ill. 60690), and "Bilirubin Standards" (American Monitor Corp., Indianapolis, Ind. 46201).

Salt-poor human albumin solutions (25 g/dl) were obtained from various sources (Merck, Sharp and Dohme, West Point, Pa. 19486; Hyland Division, Travenol Laboratories Inc., Costa Mesa, Calif. 92626; Cutter Laboratories, Berkeley, Calif. 94710; Pitman-Moore, Dow Pharmaceuticals, Indianapolis, Ind. 46206). HSA, Cohn Fraction V, was obtained from Pentex (Research Products Division, Miles Laboratories, Inc., Kankakee, Ill. 60901) and BSA, Cohn Fraction V, from Nutritional Biochemicals Corp., Cleveland, Ohio 44128. Pooled sera from hospitalized individuals were clear, nonhemolyzed, and nonjaundiced, either fresh (24 h old or less) or old (stored frozen for several months). Solutions prepared using BSA were quite turbid and were clarified by centrifugation at 4 °C at about 18 000 × *g*.

Preparation of Bilirubin Standard Solutions

Some BIL preparations, those from NBS and J. T. Baker Co., are quite insoluble in 0.1 mol/liter Na_2CO_3 and require DMSO for solubilization. BIL solutions at a concentration of 20 mg/dl were prepared either in 4 g/dl HSA, 4 g/dl BSA, or in pooled serum as follows:

Bilirubins soluble in Na_2CO_3. About 20 mg of BIL, weighed to the nearest 0.01 mg, was dissolved in 4.0 ml of 0.1 mol/liter Na_2CO_3 in a 100-ml volumetric flask. The usual precautions were observed during the preparation and assays of BIL solutions; i.e., the BIL samples were weighed, dissolved, diluted, and assayed away from windows and with the laboratory lights off. Approximately 10 to 15 min were required to obtain a red-orange clear solution. About 80 ml of protein base, adjusted to pH 7.3–7.4, was added, followed by 4 ml of 0.1 mol/liter HCl while the solution was being mixed (the exact volume of HCl is found by titrating separately the Na_2CO_3 solution with the acid to the phenolphthalein end point). The solution was diluted to volume with the protein base.

Bilirubins insoluble in Na_2CO_3. About 20 mg of BIL was suspended in 1.0 ml of DMSO in a 100-ml volumetric flask. After the BIL was uniformly dispersed, 2.0 ml of 0.1 mol/liter Na_2CO_3 was added and a clear solution was obtained within a few seconds. From this point the preparation of the standard was completed as in the preceding paragraph except that 2.0 ml of HCl was used.

The BIL standard solutions described above were diluted to various concentrations by using the appropriate diluent. Diluents were prepared exactly as the corresponding standards except that the BIL was omitted. These diluents also served as sample blanks in the subsequent analyses of the standards. The BIL solutions were analyzed immediately by the chosen methods, dispensed in polypropylene tubes (Falcon Plastics, Division of Becton, Dickinson & Co., Oxnard, Calif. 93030) and stored at −23 °C and −70 °C.

Methods

BIL analyses were performed at least in triplicate with the M-E, M-H, and J-G methods. Volumetric pipets were used throughout this study.

Malloy and Evelyn

The original M-E method was used without modification. The volumes used were: 5.0 ml of H_2O, 0.50 ml of sample or diluent (sample blank), 1.0 ml diazo reagent, and 6.0 ml of methanol—in that sequence. Thirty minutes after the reagents were mixed, absorbance measurements were made at 540 nm against a reagent blank containing 0.50 ml of water instead of sample.

Reagents

Absolute methanol. "Spectrophotometric Grade"

Gold Label" (Aldrich Chemical Co., Milwaukee, Wis. 53233).

Sulfanilic acid. Dissolve 1.0 g of reagent-grade sulfanilic acid in 200 ml of water, add 15 ml of concentrated HCl and dilute to 1 liter with water.

Sodium nitrite, 0.50 g/dl. This solution has been kept at 4 °C for 9 months without deterioration.

Diazo reagent. Mix 0.3 ml of sodium nitrite and 10 ml of sulfanilic acid solutions. This reagent was used within 1 h after its preparation.

Menes and Hogg

The volumes used were the same as those in the M-E method. After a reaction time of 10 min, absorbance measurements were made at 557 nm against a reagent blank.

Reagents

Absolute methanol. Same as in the M-E method.

Sulfanilic acid. Dissolve 5.0 g of sulfanilic acid in 500 ml of water, add 60 ml of concentrated HCl and dilute to 1 liter with water.

Sodium nitrite, 2.0 g/dl.

Diazo reagent. Mix 0.3 ml of sodium nitrite and 10 ml of sulfanilic acid solutions.

Jendrassik-Gróf

This procedure was modified by using sodium tartrate dihydrate instead of potassium sodium tartrate. With the latter reagent, crystallization of caffeine in the final reaction mixture frequently occurred, making absorbance measurements invalid. To a tube containing 4.0 ml of caffeine reagent was added 0.50 ml of sample or diluent (sample blank), followed by 1.0 ml of diazo reagent. The contents were mixed immediately and thoroughly. After 10 min, 3.0 ml of alkaline tartrate was added and mixed thoroughly by inversion. Absorbance measurements were made at 600 nm against a reagent blank containing water instead of sample.

Reagents

Caffeine reagent. Dissolve 82 g of anhydrous sodium acetate (or 125 g of $CH_3COONa \cdot 3H_2O$), 75 g of sodium benzoate and 1 g of disodium EDTA in about 500 ml of water. Add 50 g of caffeine (Aldrich Chemical Co., Milwaukee, Wis. 53233, or Eastman Kodak Company, Rochester, N. Y. 14650), stir until completely dissolved (no heating is required) and dilute to 1 liter with water. This reagent is slightly turbid but can be clarified by filtration.

Alkaline tartrate. Dissolve 75 g of NaOH and 263 g of sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) in water and dilute to 1 liter.

Sodium nitrite, 0.50 g/dl.

Sulfanilic acid. Dissolve 5.0 g of sulfanilic acid in 500 ml of water, add 15 ml of concentrated HCl, and dilute to 1 liter with water.

Diazo reagent. Mix 0.50 ml of sodium nitrite and 20 ml of sulfanilic acid solutions.

It has been reported (*21*) that the caffeine, sulfanilic acid, and alkaline tartrate reagents are stable for at least six months at room temperature.

Molar adsorptivities of the azobilirubin were calculated using the equation:

$$\epsilon = \frac{584.7 \times \text{corrected absorbance} \times \text{vol of reaction mixture in ml}}{\text{mg of bilirubin in sample analyzed}}$$

where 584.7 is the molecular weight of BIL and the corrected absorbance is the absorbance of the sample less the absorbance of the sample blank. No correction was made for the 3% decrease in volume in the final reaction mixture in the M-E and M-H methods (*15*).

Bilirubin Solutions in Chloroform

About 20 mg of BIL, weighed to the nearest 0.01 mg, was dissolved in 100 ml of $CHCl_3$ (Spectrophotometric Grade, Gold Label; Aldrich). Further dilutions were made in $CHCl_3$ and the absorbance was measured at 453 nm against $CHCl_3$ in Teflon-stoppered cuvets.

Results

Molar Absorptivity in $CHCl_3$

The molar absorptivities of BIL samples from NBS, Harleco, Pfanstiehl, and Baker were 62 000, 62 000, 60 900, and 60 400, respectively. All these values are within the specifications of the Joint Committee (*7*). The ϵ value of the NBS BIL is about 1.5% higher than that given by NBS. This discrepancy is possibly the result of evaporation of the solvent during transfer of the solution to the cuvet. It should also be pointed out that absorbance measurements at NBS were made at 25 °C, while we made no attempt to control the temperature of the cuvet compartment.

Reproducibility of the Preparation of Bilirubin Standard Solutions

Two lots of Pfanstiehl BIL were used to prepare stock standards in HSA, BSA, and pooled serum over a period of 15 months. The ϵ values obtained by the J-G method, as shown in Table 4, are essentially the same in all three protein media. They are in very good agreement with the value of 73 000 for BIL in serum reported by Gambino and Di Re (*22*). The two lots of BIL appeared to be identical. Fresh serum was used in preparations 1–4, and old frozen serum in preparations 5 through 8. Our data failed to demonstrate moderate or severe suppression of the ϵ values with old serum as reported by Gambino (*21*). Furthermore, the source of HSA does not seem to affect the ϵ values. The low coefficient of variation (<0.5%) indicates that BIL standard solutions can be prepared with good reproducibility.

Bilirubins from Various Sources

The azobilirubin ϵ values of BIL from various

Table 4. Reproducibility of the Preparation of Bilirubin Standards (Pfanstiehl Lot Nos. 6497K and 9329—J-G Method)

Preparation number	Molar absorptivity × 10^{-3a} In BSA	In HSA	In serum
1	73.4	73.7	73.7
2	73.0	73.4	72.9
3	73.9	73.5	73.4
4	73.1	74.0	73.8
5	73.6	73.4	73.2
6	73.3	73.6	73.1
7	73.3		73.6
8	73.4		73.1
9	72.7		
$\bar{X}$	73.3	73.6	73.4
SD	.35	.23	.32
CV	.47	.31	.44

[a] Molar absorptivity in all Tables is that of azobilirubin.

Table 5. Molar Absorptivities of Bilirubin from Various Sources in BSA, HSA, and Pooled Serum

Source	Molar absorptivity × 10^{-3} In BSA	In HSA	In serum
		J-G Method	
NBS	75.3	74.8	74.6
Baker	71.5	72.0	71.6
Harleco	74.0	73.9	73.2
Pfanstiehl	73.3	73.6	73.4
		M-H Method	
NBS	73.2	68.9	75.5
Baker	68.2	64.2	72.4
Harleco	72.4	69.3	75.4
Pfanstiehl	71.0	67.7	74.2
		M-E Method	
NBS	56.2	55.5	63.8
Baker	54.0	49.8	60.5
Harleco	56.7	53.5	63.0
Pfanstiehl	55.8	50.1	61.4

sources are seen in Table 5. The data are average values from analyses of at least two preparations. With the J-G and M-H methods, ϵ values were calculated from the absorbance of 20 mg/dl standards, whereas the absorbance of 10 mg/dl standards was used for calculations with the M-E method. With the J-G procedure, the product from NBS provided the highest ϵ in all protein bases. Essentially the same ϵ values were obtained for NBS and Harleco BIL with the M-H method. The results obtained with the M-E procedure were rather inconsistent. There was a variable degree of turbidity in the final reaction mixture of the M-H and M-E methods, when BIL in serum was used, raising some doubt about the validity of the ϵ values. Furthermore, ϵ values obtained by the last two methods were not very reproducible.

The molar absorptivity of azobilirubin is practically independent of the type of protein base with the J-G method. With the other two procedures, the highest ϵ values were obtained with BIL in serum. When the protein base was HSA or BSA, the molar absorptivities were substantially lower. Compared to serum, the ϵ values in HSA and BSA were on the average lower by 16% and 10%, respectively, for the M-E method. The corresponding values for the M-H procedure were lower by 9% and 4%, respectively.

Effect of Protein Concentration on Molar Absorptivity

A 20 mg/dl BIL solution was prepared in 0.1 mol/liter Na_2CO_3. From this solution 15 mg/dl standards with an HSA concentration from 1 to 6 g/dl were prepared as rapidly as possible. In addition, the original stock solution was diluted to 15 mg/dl with 0.134 mol/liter phosphate buffer, pH 7.4. All solutions were analyzed by the J-G method, and the results are shown in Table 6. A minimum HSA concentration, between 1 and 2 g/dl, is required to obtain maximum absorbance.

Linearity

The linearity of the J-G method is illustrated in Tables 7 and 8. When a high BIL standard in serum was diluted with serum diluent (Table 7), the linearity was excellent as judged by the essentially constant value of the ϵ. In contrast, high dilutions of the same standard with saline resulted in a significant suppression of the ϵ values. This experiment was repeated several times with very consistent results, which are in disagreement with the statement made by Fog (*23*) that a fifty-fold dilution of protein has no effect on the molar absorptivity. Good linearity was also observed with BIL in HSA when the same

Table 6. Effect of HSA Concentration on the Molar Absorptivity (Pfanstiehl Bilirubin—J-G Method)

Medium	Molar absorptivity × 10^{-3}
0.1 mol/liter Na_2CO_3	70.4
Phosphate buffer, pH 7.4	71.4
HSA, g/liter	
1	72.8
2	73.6
3	73.5
4	73.4
5	74.0
6	73.7

Table 7. Effect of Bilirubin Concentration on the Molar Absorptivity (Bilirubin in Serum—J-G Method)

Concentration, mg/dl	Molar absorptivity × 10^{-3}, Diluent: Serum	Saline
0.50	74.0	64.6
1.0	73.7	68.2
2.0	73.1	71.6
4.0	73.1	72.4
10.0	72.4	72.6
20.0	73.1	—

Table 8. Effect of Bilirubin Concentration on the Molar Absorptivity (Bilirubin in HSA or BSA—J-G Method)

	Molar absorptivity × 10^{-3}	
	Diluent	
Concentration, mg/dl	HSA	BSA
0.50	73.2	69.3
1.0	74.6	70.6
5.0	—	72.8
10.0	74.1	72.6
20.0	74.3	72.8

protein was used as diluent, while slight deviations from linearity occurred with BIL in BSA (Table 8). The use of saline as diluent for BIL solutions in HSA and BSA has about the same effect as for BIL in serum. The alkaline azobilirubin follows Beer's law up to an absorbance of 1.7 and perhaps higher.

Similar experiments were conducted with the M-H and M-E methods. The linearity was good for both methods only with BIL standards in serum, and when serum diluent was used for the dilution of a stock (20 mg/dl) BIL solution. Dilutions with saline had the same effect as that seen with the J-G procedure. Serious deviation from linearity was observed with both methods when either HSA or BSA were used as the protein bases. The ϵ values were severely suppressed near the physiologic range even when dilutions were made with the appropriate diluents.

Precision

The precision of the methods was determined at different BIL concentrations. Each sample was consecutively analyzed by the same individual. The data in Table 9 indicate that the precision is very good for all methods, even at BIL concentrations near the normal range. With the M-E method, the concentration of the high standard was 10 mg/dl.

Caffeine Reagent

Caffeine reagents were prepared with caffeine from two different sources. BIL solutions analyzed with both reagents provided identical ϵ values. Caffeine obtained from Eastman was recrystallized from boiling water. There was no change in the ϵ of azobilirubin when the recrystallized material was used.

The caffeine reagent, an almost saturated solution, has an undesirably high viscosity. The necessity of such a concentrated reagent in the BIL determination by the J-G method has not been documented. BIL solutions (20 mg/dl) in HSA, BSA, and in pooled serum were analyzed with caffeine reagent of variable concentration. The data in Table 10 indicate that the absorptivity of azobilirubin remains essentially constant even when this reagent is diluted to 35% of its original strength. Further dilution results in a drastic drop of the ϵ value of BIL in serum. Although a more dilute reagent may be quite satisfactory, the full-strength reagent was used throughout this study.

Table 9. Precision of Various Methods in Bilirubin Analysis (Bilirubin in Serum or HSA)

	J-G		M-H	M-E
N = 10	In serum	In HSA	In serum	In serum
0.5 mg/dl				
$\bar{X}$	0.0372	0.0354	0.0276	0.0198
S	0.0004	0.0005	0.0006	0.0004
CV	1.1	1.3	2.3	2.2
$\epsilon \times 10^{-3}$	74.0	72.6	80.7	58.0
1 mg/dl				
$\bar{X}$	0.0746	0.0721	0.0503	0.0400
S	0.0003	0.0002	0.0007	0.0008
CV	.39	.34	1.4	2.0
$\epsilon \times 10^{-3}$	74.1	73.9	73.6	58.5
20 mg/dl				
$\bar{X}$	1.478	1.436	1.008	0.0414[a]
SD	0.0022	0.0014	0.0040	0.0045
CV	.15	.10	.40	1.1
$\epsilon \times 10^{-3}$	73.5	73.6	73.7	60.1

[a] Concentrations: 10 mg/dl.

Table 10. Effect of the Concentration of Caffeine Reagent on the Molar Absorptivity (J-G Method)

Reagent concentration, % of full strength	Molar absorptivity × 10^{-3} In HSA	In BSA	In serum
100	71.8	73.0	73.7
90	71.8	72.9	73.8
70	72.3	72.9	73.6
50	72.4	73.4	74.2
35	72.7	74.0	73.8
25	72.0	73.1	57.9

The incorporation of EDTA in the caffeine reagent was proposed by Holtz and van Dreumel (*24*) in order to avoid complexes of azobilirubin with heavy metal ions that interfere in the BIL measurement. We have observed some deviation from linearity at low BIL levels when the EDTA was omitted.

When K,Na-tartrate was used in the alkaline buffer, a variable degree of turbidity was occasionally seen in the final reaction mixture. The mixture was centrifuged and the sediment was dissolved in $CHCl_3$. Upon evaporation of the solvent, a crystalline substance was obtained that had an ultraviolet absorption spectrum identical to that of caffeine. Turbidity was never observed with Na-tartrate in the alkaline buffer.

Dyphylline [7-(2,3-dihydroxypropyl)-theophylline] has been used instead of caffeine (*2*), to avoid the turbidity that sometimes occurs in the reaction mixture of the J-G method. Michaëlsson (*2*) reported that the absorptivity of azobilirubin and the stability of color were the same with both xanthine derivatives. We have repeated his experiment and confirmed his findings. Thus dyphylline and caffeine are perhaps equally suitable as accelerators, except that the former causes excessive foaming (a solid-type foam) in the reaction mixture, which may be considered undesirable.

Sodium Nitrite

There is a lack of agreement with regard to the stability of the 0.50 g/dl $NaNO_2$ solution at 4 °C. A solution of $NaNO_2$ was kept for nine months in the refrigerator and was periodically compared with a fresh solution in the BIL analyses. The preparation remained stable throughout this period.

Diazo Reagent

Gambino and Di Re (*22*) have challenged the myth about the 30-minute stability of the diazo reagent. They reported that this reagent is stable for 24 h. We have confirmed their findings, and found that the diazo reagent used with the J-G method is stable either at room temperature or at 4 °C for at least 72 h. However, it was routinely used within an hour after preparation.

Commercial Control Preparations

Lyophilized commercial controls were reconstituted according to the manufacturer's directions and analyzed at least in triplicate. Sample blanks were prepared by substituting sulfanilic acid for the diazo reagent.

The data in Table 11 indicate serious discrepancies between the listed and found values for some control sera. The largest difference was observed with "Calibrate 3" (Lot A) and "Metrix." An unusual phenomenon was observed in the analysis of "Metrix." The color development was slow and at the end of the 10-min coupling period the solution had an orange tint. When the coupling time was increased to 45 min, a higher absorbance was obtained. Upon centrifugation of the reconstituted material, a gelatinous orange precipitate was obtained, indicating that some BIL was not in solution. The precipitate was washed with water, centrifuged, dissolved in 0.1 mol/liter Na_2CO_3, and analyzed by the J-G method. The amount of BIL present in the precipitate was 0.029 mg per vial of "Metrix," or 14% of the amount listed by the manufacturer. When the same control was analyzed by the M-H method, a BIL value of 22.1 mg/dl was obtained, indicating that "Metrix" contains more bilirubin than listed by the manufacturer.

One lot of BIL standards from American Monitor Co. provided values close to those listed. A different lot, however, gave values that were, with one exception, about 0.10% less (Table 12). Finally, some BIL controls were analyzed simultaneously by the J-G and M-H methods, and the results are shown in Table 13. The different values of the American Monitor preparations obtained by the two methods are expected, since the BIL is in BSA base and our standard with the M-H method was BIL in serum. The results obtained for "Calibrate 3," "Versatol," and "Monitrol II" are difficult to explain. It is evident that the BIL values in these controls are dependent on the methodology.

Table 11. Bilirubin Content of Commercial Controls (J-G Method)

Control	Listed value (mg/dl)	Found value (mg/dl)	Difference, %
Monitrol II	3.6	3.7	+2.8
Calibrate 3 Lot A	5.0	4.1	−18.0
Calibrate 3 Lot B	5.0	4.5	−10.0
Versatol Pediatric	20.7	18.9	−8.7
Metrix	20.7	16.7	−19.3
Metrix[a]	20.7	18.1	−12.5
Dade BIL Control	20.0	19.4	−3.0
American Monitor D	10.0	9.7	−3.0
American Monitor E	15.0	14.9	−0.7
American Monitor F	20.0	19.6	−2.0

[a] Coupling time: 45 min.

Table 12. Bilirubin Content of American Monitor Standards (J-G Method)

Vial	Listed value (mg/dl)	Found value (mg/dl)	Difference, %
A	1.0	0.9	−10.0
B	3.1	2.8	−9.7
C	5.0	4.7	−6.0
D	10.0	9.1	−9.0
E	15.0	13.5	−10.0
F	20.0	18.1	−9.5

Table 13. Bilirubin Content of Commercial Controls by M-H and J-G Methods

Control	Listed value	Found value	
		M-H	J-G
	mg/dl		
Calibrate 3	5.0	4.1	4.5
Pediatric Versatol	20.7	18.1	18.8
Monitrol II	3.5	3.3	3.5
American Monitor D	10.0	8.7	9.4
American Monitor F	18.9	17.1	18.1

Stability of Frozen Bilirubin Solutions

Frozen BIL solutions, 20 mg/dl, were kept for five months at −23 °C and were analyzed several times during this period by the J-G method. The results from this experiment are given in Table 14. There is a significant deterioration of BIL with storage in both the 4 and 8 g/dl BSA solutions, 2% and 1.5% per month, respectively.

Data on the stability of BIL in HSA, BSA, and pooled serum, at −70 °C, are shown in Tables 15 and 16. The BIL concentration remained essentially unchanged over a period of seven months when HSA or BSA were used as the protein base. Bilirubin in pooled serum seems to deteriorate a little faster than in the other two protein media.

Discussion

The results of this study provide an explanation for the lack of accuracy in the determination of BIL and identify some possible sources of error.

Table 14. Stability of Bilirubin Solutions at −23 °C (Pfanstiehl Bilirubin in BSA Solutions—(J-G Method)

	Molar absorptivity × 10^{-3}	
Date	In 4 g BSA/dl	In 8 g BSA/dl
10-15-71	73.0	73.4
10-21-71	72.6	72.4
10-29-71	72.1	72.2
11-5-71	—	71.9
11-9-71	71.6	72.2
11-23-71	70.8	71.1
12-9-71	71.2	71.6
2-2-72	70.0	69.2
4-10-72	66.3	68.0

Table 15. Stability of Bilirubin Solutions at −70 °C (Pfanstiehl Bilirubin in HSA and Pooled Serum—J-G Method)

	Molar absorptivity × 10^{-3}	
Date	In 4 g HSA/dl	In serum
12-27-71	73.7	73.7
2-1-71	73.4	72.9
3-7-72	73.2	71.7
4-10-72	73.2	72.3
9-14-72	72.4	71.2
10-27-72	71.4	—

Table 16. Stability of Bilirubin Solution at −70 °C (NBS Bilirubin in 4 g HSA or BSA per Deciliter—J-G Method)

	Molar absorptivity × 10^{-3}	
Date	In HSA	In BSA
3-16-72	73.9	74.1
5-9-72	74.3	—
6-19-72	74.2	74.7
10-27-72	73.2	73.8

Standardization

The lack of accuracy of BIL determinations can be attributed to a great extent to inadequate standardization, because the precision of the three methods is good even at concentrations near the physiologic range (Table 9). Bilirubin standards have been prepared in HSA (*12, 13*), BSA (*10, 14*), or pooled serum (*2, 7, 9, 11*), and commercial BIL controls and "standards" are prepared in all of the above protein bases. The Dade BIL control is prepared, according to the manufacturer, in crystalline HSA; the American Monitor "standards" in BSA; and all the other examined controls in pooled serum. Even when the standards are accurate, the possibility of error exists and depends on the choice of method and the protein base used in the preparation of the standard. It is apparent that with the M-E and M-H methods the *only* suitable protein base is pooled serum; serum BIL will be overestimated if standards in HSA or BSA are used. Compared to BIL in serum, the ϵ values of azobilirubin in BSA and HSA are substantially lower. In contrast, the molar absorptivities of azobilirubin with the J-G method are almost identical in all three protein bases. The preparation of BIL standards has been inconvenient, and Michaëlsson et al. (*25*) have recommended the use of the molar absorptivity of azobilirubin as a means for standardization. There are various reasons for the reluctance of many laboratories to prepare BIL standards. Among these reasons are: differences in purity of the BIL preparations encountered in the past, the lack of a preparation with known purity, the difficult solubilization in Na_2CO_3, and the meager information available with regard to the stability of the prepared standards.

Pure BIL is now available from the NBS, and should be used to ascertain the purity of BIL from commercial sources. Bilirubins that appear amorphous under the microscope (Harleco, Pfanstiehl) dissolve rather easily in 0.1 mol/liter Na_2CO_3 in about 10 to 15 min. We have confirmed, however, the experience of Dybkaer and Hertz (*9*) that it is impossible to prepare a 20 mg/dl standard according to the recommendation of the Joint Committee (*7*) and Gambino (*21*); i.e., to dissolve 20 mg of BIL in 2 ml of Na_2CO_3. Solution can be effected by using twice the specified volume. Crystalline bilirubins (from NBS or J. T. Baker) are relatively insoluble in Na_2CO_3. Insoluble material was present 2 h after the addition of Na_2CO_3. However, if the material is first suspended in DMSO, it dissolves immediately upon addition of Na_2CO_3. The use of the two solvents has several advantages: (*a*) it permits the use of the NBS BIL; (*b*) it reduces the time BIL remains at a high pH; (*c*) it assures that all the BIL is in solution, a fact that is determined with great difficulty if Na_2CO_3 only is used; and (*d*) the protein base can be added directly to the BIL solution without the risk of protein precipitation. The presence of DMSO does not change the molar absorptivity of azobilirubin. HSA and BSA are preferable to serum as the protein base because they contain no detectable BIL, which is invariably present in pooled sera. The resulting BIL solutions are crystal clear, and the same protein solutions can be used to prepare working standards with a constant protein concentration. With the J-G method, HSA or BSA is the preferred protein base. Dybkaer and Hertz (*9*) have recently described a technique for preparing BIL standards. We found their procedure unusually, and perhaps unnecessarily, complicated.

Unlike other primary standards, BIL solutions should be assayed and the molar absorptivity calculated before they are used in the laboratory. The ϵ of the azobilirubin can perhaps provide, as suggested by several investigators, a better criterion of purity than that of BIL in $CHCl_3$. There is a need for a collaborative study to establish the molar absorptivities of azobilirubin for the most accepted methods with certified NBS BIL. The range of ϵ in $CHCl_3$ (60 700 ± 1 600) for an acceptable BIL is too wide, and a more narrow range can be established for azobilirubin, as evidenced by the data in Table 4.

BIL solutions deteriorate appreciably when stored at −23 °C. This deterioration, about 2% per month, prohibits long storage at this temperature. BIL solutions ranging from 5 to 20 mg/dl were stored at −16 °C for 12 days. There was an almost uniform 4% deterioration at the end of this period. At −70 °C, BIL solutions show good stability. The rate of deterioration is so slow that we have been unable to detect any change over a period of seven months.

Methods

The purpose of this communication is not to propose the adoption of a certain procedure. We would like, however, to make some comments in regard to the methods examined. The J-G procedure is characterized by an excellent precision, both at normal and abnormal levels (Table 9). The rate of color development is very rapid. Upon addition of the diazo reagent to a caffeine-BIL mixture, 99.5% of the maximum absorbance is obtained in less than 30 s. Reagents prepared with different lots of materials provided the same ϵ values. The color produced adheres to Beer's law up to an absorbance of 1.7 and perhaps higher. The sensitivity is considerably higher than by the other two methods. The inhibition by hemoglobin is almost completely eliminated by the use of ascorbic acid (*25*).

It has been claimed (*1*) that complete reaction of all the protein-bound BIL cannot be achieved by ordinary caffeine and benzoate mixtures. However, results reported by the CDC Proficiency Testing (Clinical Summary Chemical Analysis, May 12, 1972) tend to dismiss this claim. Procedures in which methanol is used as the accelerator showed a negative bias on all samples of the survey. The values obtained by the M-E method were 55 to 90% of those of the J-G procedure.

There are two reports (*26, 27*) describing an inhibitory effect of dyphylline on the determination of total BIL. Our experience with dyphylline is limited, and this point requires further investigation. At the present time, the J-G procedure is the most thoroughly investigated (*2, 3, 21, 25*), and because its advantages outweigh the disadvantages it appears to be the method of choice.

The M-E method suffers from several inadequacies. It has been reported by Michaëlsson (*2*) that at high unconjugated BIL concentrations color development requires more than 30 min, and that the absorptivity of axobilirubin depends on the "age" of methanol; a newly opened bottle provided higher ϵ values than a repeatedly used bottle. Hargreaves (*28*) stated that it was impossible to measure accurately the BIL content of certain neonatal sera, even when the reaction time was increased to 60 min, and that accurate results were obtained only when a higher dilution of serum was used. According to Henry (*29*), samples must be diluted when the BIL concentration exceeds 15 mg/dl. The variable turbidity in the reaction mixture and the strong inhibitory effect of hemoglobin (*2*) are serious drawbacks. The method is considerably less sensitive than the J-G procedure. If the volume of sample is kept constant, under the conditions of our studies a 10 mg/dl NBS BIL standard in serum will give absorbances of 0.749, 0.517, and 0.425 with the J-G, M-H, and M-E methods, respectively. Only pooled serum may be used for standardization, and the ϵ values for azobilirubin are not very reproducible from day to day. The latter can be attributed either to turbidity or to the quality and volatility of methanol.

The M-H procedure possesses two distinct advantages over the M-E method. Color development is complete in 10 min, even when the BIL concentration is as high as 20 mg/dl, and its sensitivity is higher by 25%. All other comments made for the M-E method are applicable to the M-H procedure.

It has been recommended that standardization in the M-E and M-H methods be carried out with BIL dissolved in a mixture of $CHCl_3$ and CH_3OH (*5, 15 16*). There are several objections to this approach. The standardization is performed in a protein-free medium and the absorptivity of BIL may be different in the absence of protein. Henry (*30*) reported that the ϵ value of azobilirubin, with the M-E method, is substantially higher in the absence of protein. Furthermore, BIL in a mixture of $CHCl_3$ and CH_3OH is extremely unstable, and such solutions must be used within 2 to 4 min after their preparation (*16*). For these reasons it appears preferable to standardize the M-E and M-H methods with BIL in serum as recommended by the Joint Committee (*7*).

Commercial Controls

Commercial controls can be improved if manufacturers take some corrective steps. It is important that commercial BIL controls be assayed against the NBS BIL standard. It is also important to assure that all BIL is in solution before the protein base is added. If the protein base is other than serum, the assigned BIL values will vary with the analytical method unless the J-G procedure is used. When controls are made in HSA or BSA, the user must be informed that these are not suitable for the M-E and M-H procedures. They can be used only when the assigned values are established with a reference standard of BIL in serum. To be more explicit, separate listed values should be given for the various methods. The importance of accurate BIL determinations in neonates cannot be overemphasized, since the concentration of BIL is the major criterion for exchange transfusion therapy.

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Addendum

Since this paper was accepted for publication, we have observed a suppression of the molar absorptivity of azobilirubin, by the J-G method, in a BIL standard in serum.

An NBS BIL standard in fresh pool sera gave an ϵ value for azobilirubin of 71.1×10^3 as compared to 74.6×10^3 previously observed. To exclude an error in the preparation of the standard solution, aliquots of the standard were diluted with 0.134 mol/liter phosphate buffer, pH 7.4, and absorbance was measured at 462 nm against a similarly treated serum diluent. The molar absorptivity of BIL was 49.8×10^3, which agrees very well with the value of 50.0×10^3 that we have observed in the past.

Deterioration of the NBS material was also ruled out by preparing a BIL standard in HSA on the same day that the standard in serum was made. The ϵ value for azobilirubin of this preparation was 74.8×10^3, which is identical to that shown in Table 5.

References

1. Mather, A., Reliability of bilirubin determinations in icterus of the newborn infant. *Pediatrics* **26**, 350 (1960).

2. Michaëlsson, M., Bilirubin determination in serum and urine. *Scand. J. Clin. Lab. Invest.* **13**, *Suppl. 56*, 1 (1961).

3. Nosslin, B., The direct reaction of bile pigments in serum. Experimental and clinical studies. *Scand. J. Clin. Lab. Invest.* **12**, *Suppl. 49* (1960).

4. Jendrassik, L., and Gróf, P., Vereinfachte photometrische Methoden zue Bestimmung des Blutbilirubins. *Biochem. Z.* **297**, 81 (1938).

5. Malloy, H. T., and Evelyn, K. A., The determination of bilirubin with the photoelectric colorimeter. *J. Biol. Chem.* **119**, 481 (1937).

6. Meites, S., and Hogg, C. K., Studies on the use of the van den Bergh reagent for determination of serum bilirubin. *Clin. Chem.* **5**, 470 (1959).

7. Joint Committee Report. Recommendation on a uniform bilirubin standard. *Clin. Chem.* **8**, 405 (1962).

8. With, T. K., Neonatal jaundice. *Lancet* **ii**, 618 (1962).

9. Dybkaer, R., and Hertz, H., A reference for determination of bilirubin concentration in serum using bilirubin in cyanide-formamide for enrichment of serum. *Scand. J. Clin. Lab. Invest.* **25**, 151 (1970).

10. Gadd, K. G., Preparation of bilirubin standards. *J. Clin. Pathol.* **19**, 300 (1966).

11. Billing, B., Haslam, R., and Wald, N., Bilirubin standards and the determination of bilirubin by manual and Technicon AutoAnalyzer methods. *Ann. Clin. Biochem.* **8**, 21 (1971).

12. Shinowara, G. V., Spectrophotometric studies on blood serum and plasma. The physical determination of hemoglobin and bilirubin. *Amer. J. Clin. Pathol.* **24**, 696 (1954).

13. Jackson, S. H., and Hernandez, A. H., A new "bilirubinometer" and its use in estimating total and conjugated bilirubin in serum. *Clin. Chem.* **16**, 462 (1970).

14. Simmons, N. A., An automated method for serum bilirubin determination. *J. Clin. Pathol.* **21**, 196 (1968).

15. Meites, S., and Traubert, J. W., Use of bilirubin standards. *Clin. Chem.* **11**, 691 (1965).

16. Carter, R. E., and McGann, C. J., The standardization of the van den Bergh reaction for serum bilirubin. *Clin. Chem.* **5**, 106 (1959).

17. Sims, F. H., and Horn, C., Some observations on Powell's method for the determination of serum bilirubin. *Amer. J. Clin. Pathol.* **29**, 412 (1958).

18. Rand, R. N., and di Pasqua, A., A new diazo method for the determination of bilirubin. *Clin. Chem.* **8**, 570 (1962).

19. Helman, E. Z., Reingold, I. M., and Gleason, I. O., Plea for standardization of commercial calibration materials for automated instruments. *Clin. Chem.* **17**, 1144 (1971).

20. Laessig, R. H., Schwartz, T. H., and Paskey, T. A., Cooperative survey of "SMA" analyzers in Wisconsin. A method of improving interlaboratory performance. *Clin. Chem.* **19**, 62 (1973).

21. Gambino, R. S., Bilirubin (Modified Jendrassik and Gróf). In *Standard Methods of Clinical Chemistry,* **5**; S. Meites, Ed. Academic Press Inc., New York, N. Y., 1965, p 55.

22. Gambino, R. S., and Di Re, J., *Bilirubin assay.* American Society of Clinical Pathologists, Chicago, Ill., 1968.

23. Fog, J., Determination of bilirubin in serum as alkaline "azobilirubin." *Scand. J. Clin. Lab. Invest.* **10**, 241 (1958).

24. Holtz, A. H., and van Dreumel, H. J., Stabilization of azobilirubin solution by EDTA. *Clin. Chim. Acta* **20**, 355 (1968).

25. Michaëlsson, M., Nosslin, B., and Sjölin, S., Plasma bilirubin determination in the newborn infant. *Pediatrics* **35**, 925 (1965).

26. Thompson, R. P. H., Modification of Michaëlsson's method for the measurement of plasma total bilirubin. *J. Clin. Pathol.* **22**, 439 (1969).

27. Baer, D. M., and Wood, D. C., Artifactual lowering of the total bilirubin value. *Clin. Chim. Acta* **71**, 1 (1967).

28. Hargreaves, T., Neonatal serum bilirubin estimation. *Clin. Chim. Acta* **26**, 331 (1969).

29. Henry, R. J., In *Clinical Chemistry: Principles and Technics.* Hoeber, New York, N. Y., 1964, p 590.

30. Henry, R. J., In *Clinical Chemistry: Principles and Technics.* Hoeber, New York, N. Y., 1964, p 585.